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FINAL REPORT

NEURAL STEM CELL DIFFERENTIATION UNDER ELECTRIC STIMULATION

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The following report represents a description of progress made during the one-year funding period of NJCSCR fellowship. The objective of this project is to examine and optimize neural stem cell differentiation in an electrically charged environment. Ultimately large numbers of functionally stable cells differentiated from embryonic stem cells using this approach can be incorporated into cell-based therapies for studies in spinal cord injury. To achieve these goals, we have been collaborating with Dr. Bonnie Firestein from the Department of Cell Biology and Neuroscience and Dr. Dajun Zhang from the Department of Mechanical and Aerospace Engineering at Rutgers.

Specific Aim 1: To develop a differentiation strategy for generation of neural progenitor cells from embryonic stem cells in vitro.

Many previous studies have established techniques to differentiate stem cells into neuronal lineages cells and/or promote neuron extension. Nevertheless a heterogeneous population oftentimes arises. The studies proposed in Aim 1 were designed to develop a differentiation method for generating a homogenous population of neural progenitor cells.

Neuronal differentiation and enrichment via EB culture: We have adapted the hanging drop technique to generate EBs from the ES cell line, ES-D3 (ATCC, Manassas, VA). In the presence of retinoic acid, we successfully induced 33% neurofilament expressing neuronal cells as early as Day 8. Based on the immunofluorescent intensity level of neurofilament marker, the optimal concentration of retinoic acid was found to be 10^{-7}M. In the retinoic acid induced system, differentiated cells expressed a variety of neural markers. Among these cells, over 60% expressed progenitor cell marker A2B5. In an effort to isolate committed subpopulation as a source of transplantable cells following nerve injuries, neuronal progenitor cells were isolated using two-color fluorescence-activated cell sorting (FACS) using A2B5 and PSA-NCAM antibodies on Day 8. It was previously demonstrated by Schmandt et al. that this method was able to yield a neuronal population at purity of over 95% after sorting, and can be used a high purity selection of early ES cell derived neurons. These cells were maintained in Iscove's Modified Dulbecco's medium supplemented with 20% fetal bovine serum, 4 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin and 10 µg/mL gentamicin (all from Gibco) after sorting. In one month culture period, sub-populations continued to proliferate. While expressions of progenitor markers such as A2B5 and PSA-NCAM declined over time, they continued to express more mature markers such as NF, O1 and GFAP. Hence these cell sub-populations shifted to a more mature differentiation stage after the sorting.

Alginate encapsulation: Due to the important concern that large numbers of functionally stable cells are required for stem cell replacement therapy, a more scalable differentiation method is necessary. An alginate culture system was therefore developed. The encapsulation system implements an electrostatic droplet approach, and resulted in a bead diameter of 500 µm at an applied voltage of 6.4 kV. We were able to maintain a solid bead core, thus maintaining the property of a single cell suspension. Cells were recovered by depolymerizing

alginate capsules in sodium citrate solution. Previous studies in our laboratory demonstrated that the alginate microenvironment was shown to maintain cell viability, to be conducive to spontaneous ES cell differentiation to hepatocyte lineage cells, and to maintain differentiated hepatocyte cellular function. Thus far we have demonstrated the spontaneous differentiation of both hepatocyte and neuronal lineage cells in this system. In the absence of retinoic acid only 5% cells express NCAM during the first 14 days post encapsulation. As hepatocyte differentiation increases, NCAM expression disappears. However, in the presence of 10^{-7}M retinoic acid, the differentiation progression is altered. Neuronal lineage cells persist throughout the 20 day encapsulation period. The percentage of cells positively stained for A2B5, NF 150kD, O1 and GFAP markers were 24.7%, 41.9%, 32.3% and 38.6% respectively. As compared to traditional EB culture, it was shown that about 38% of cells stained positive for neurofilament marker on Day 8 in the presence of retinoic acid. These studies demonstrate that the efficiency of neuronal lineage differentiation can be duplicated within the alginate microenvironment. The alginate encapsulation system provides an alternative differentiation environment to test a range of soluble and electrical environments to control neuronal lineage differentiation.

Specific Aim 2: To establish a culture system for neural progenitor cell maturation under the influence of electric fields.

Design of silicone microchannel device: The studies proposed in Aim 2 were designed to incorporate both cultivation and electric field into neural differentiation system. Our group has previously designed and fabricated a microchannel network for axonal guidance. The microfluidic network is fabricated in polydimethylsiloxane (PDMS, a transparent silicon polymer), and can be integrated with multiple electrode arrays (MEAs) for electrophysiological detection. Electrodes can be treated with gelatin and other ECM proteins for cell attachment. Once the MEA and the PDMS microchannel surfaces activated with oxygen plasma, they are brought into alignment and permanently bound to each other. The electrodes will be connected to an external power supply (HP Agilent E3612A). This integrated multiple electrode array will be adapted in Aim 2 for generating electrical fields. ES cells or EBs will be placed into the wells in the PDMS and allowed to stabilize in culture for overnight, when the stimulation will be performed.

Electric stimulation of PC 12 cells and ES cells: Instead of using murine neuronal-like cell line, Neuro-2a (ATCC CCL-131) as described in the proposal, we have studied the effect of electrical stimulation on the differentiation with rat neuronal cell line PC12 as our first attempt. These studies indicate that we can increase neuronal extension in the presence of nerve growth factor and electrical fields. We have also applied electrical field stimulation to embryoid bodies and have observed an effect on cell distribution on the periphery of the differentiating embryoid body. By Day 8, the diameter of embryoid body increased by 18% with electric treatment, in which the cells tend to migrate and grow away from EB cores. These studies indicate that we can change the spatio-temporal interaction in the developing EB, which is important in regulating lineage commitment.

Specific Aim 3: To apply the approach developed in Aim 2 to embryonic cells for a more efficient and higher yielding differentiation technique

The studies proposed in Aim 3 were designed to apply electric stimulation to increase yield of cells committed to neural progenitor and further enhance neural functions. To investigate the effectiveness of the electric stimulation, we will utilize the integrated multiple electrode arrays described in Aim 2. We have targeted this goal in three ways:

(1) We have demonstrated in Aim 1 that we could isolate early oligodendrocyte precursors (i.e. A2B5 expressing cells) using FACS, and maintain them in secondary culture for more than 30 days. In Aim 3, sorted and unsorted cells differentiated from embryoid body mediated culture will be replated in PDMS wells and allowed to attach overnight. Then they will be exposed to electric filed in the pulsing buffer containing 255mM sucrose, 1mM $CaCl_2$, 1mM $MgCl_2$ and 5mM HEPES (pH 7.2) as described above. An electric field with field strengths ranging from 100 V/m to 750 V/m and duration of 30 seconds to 120 seconds will be applied to sorted and unsorted cells. However, this range may be extended to higher levels if necessary. The yield of neural progenitor cells will be evaluated using FACS and immunofluorescent staining.

(2) To further enhance and maturate differentiated cells, retinoic acid could be supplemented during first 8 days in embryoid body mediated culture, and follow the experimental design in Aim 3(1).

(3) To scale up the differentiated cell output, we would also adapt alginate encapsulation differentiation system in Aim 1, with and without of cell sorting technique using A2B5/PSA-NCAM as described above.

While these studies are still ongoing, this approach will provide a valuable option for intracellular regulation of neuronal differentiation in the presence and absence of electrical field stimulation.